Original Research Article

Dietary N-carbamylglutamate or L-arginine supplementation improves hepatic energy status and mitochondrial function and inhibits the AMP-activated protein kinase-peroxisome proliferator-activated receptor γ coactivator-1α-transcription factor A pathway in intrauterine-growth-retarded suckling lambs

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ABSTRACT

The objective of this study was to investigate the effects of dietary administration of L-arginine (Arg) or N-carbamylglutamate (NCG) on hepatic energy status and mitochondrial functions in suckling Hu lambs with intrauterine growth retardation (IUGR). Forty-eight newborn Hu lambs of 7 d old were allocated into 4 treatment groups of 12 lambs each, in triplicate with 4 lambs per replicate (2 males and 2 females) as follows: CON (lambs of normal birth weight, 4.25 ± 0.14 kg), IUGR (3.01 ± 0.12 kg), IUGR + 1% Arg (2.99 ± 0.13 kg), or IUGR + 0.1% NCG (3.03 ± 0.11 kg). The experiment lasted for 21 d, until d 28 after birth, and all lambs were fed milk replacer as a basal diet. Compared with IUGR lambs, NCG or Arg administration increased ($P < 0.05$) the adenosine triphosphate (ATP) level and the activities of complexes I/III/IV, isocitrate dehydrogenase and citrate synthase in the liver. Compared with CON lambs, the relative mRNA levels of adenosine monophosphate-activated protein kinase α1 (AMPKα1), peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) and transcription factor A (TFAM) were increased ($P < 0.05$) in the liver of IUGR lambs, but were decreased ($P < 0.05$) in the liver of NCG- or Arg-treated lambs compared with those in the IUGR lambs. Compared with IUGR lambs, NCG or Arg administration decreased ($P < 0.05$) the total AMPKα (tAMPKα)-to-phosphorylated AMPKα (pAMPKα) ratio and the protein expression of PGC1α and TFAM. The results suggested that dietary Arg or NCG supplements improved hepatic energy status and mitochondrial function and inhibited the AMPK-PGC1α-TFAM pathway in IUGR suckling lambs.

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1. Introduction

Intrauterine growth restriction (IUGR) is a serious condition in livestock production, as fetal growth restriction is associated with long-term limitations in health and performance in postnatal life (Eke et al., 2019). IUGR animals experience higher rates of neonatal morbidity/mortality and lower postnatal growth rate and

reproductive performance (Hales et al., 1991). Moreover, IUGR has been shown to impair the mitochondrial biogenesis and hepatic oxidative phosphorylation in rats (Park et al., 2003) and piglets (Zhang et al., 2016a, 2017).

Timely nutritional interventions could alleviate the adverse metabolic consequences of IUGR. L-Arginine (Arg), a nutritionally semi-essential amino acid involved in multiple biological functions, has been shown to increase gluconeogenesis and trigger the hormonal regulation of energy metabolism (Tangara et al., 2010; Wu et al., 2009). Dietary Arg or N-carbamylglutamate (NCG) supplementation has been shown to alleviate IUGR-induced hepatic oxidative damage in newborn lambs via activating the nitric oxide (NO) pathway as well as the antioxidative and phase II metabolizing enzymes (Zhang et al., 2020). It is also well-known that Arg can promote mitochondrial biogenesis and functions via the NO signaling pathway (Nisoli et al., 2003), which is one of the pathways involved in the intracellular redox environment modulation (Yin et al., 2014). As an analogue of N-acetylglutamate synthase (NAG), NCG is metabolically stable and can boost the Arg content in plasma and enhance its endogenous synthesis via the activation of intestinal carbamoyl phosphate synthase-1 and pyrroline-5 carboxylate synthase (Wu et al., 2004).

Mitochondria are the main organelle that produces reactive oxygen species (ROS) and adenosine triphosphate (ATP), which exerts an important role in regulating cellular oxidative injury (Zhang et al., 2018a). The function of mitochondria is closely related to the availability of nutrients (Theurey et al., 2016). Hepatic mitochondrial dysfunction and oxidative injury have been reported in IUGR pigs (Zhang et al., 2018b) and heat-stressed broilers (Zhang et al., 2018c). However, little information is available on the role of dietary Arg or NCG administration on hepatic mitochondrial function in IUGR lambs.

The adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) and silent information regulator 1 (SIRT1) are recognized to play vital roles in regulating cellular energy metabolism (Chau et al., 2010). Additionally, PGC1α, the upstream factor of the nuclear respiratory factors 1 and 2 (NRF1/2) and the transcription factor A mitochondrial (TFAM) can increase the biogenesis of mitochondria, and thus can be used as biomarkers to sense nutrient availability (Rodgers et al., 2005). However, the regulatory mechanism regarding hepatic mitochondrial metabolism in IUGR suckling lambs supplemented with NCG or Arg is still unclear.

Therefore, the current study aimed at investigating the effects of dietary NCG or Arg supplementation on hepatic energy status and mitochondrial function and linking these responses to AMPK-PGC1α-TFAM pathway activation in the liver of IUGR suckling Hu lambs.

2. Materials and methods

All experimental protocols were approved by the Animal Care and Use Committee of the Yangzhou University (SXXY, 2015-0054) and were performed following the practical animal protection law and the Guide for the Care and Use of Laboratory Animals formulated by the National Research Council (China).

2.1. Animals and treatments

Suckling Hu lambs were identified as IUGR when the birth weights were ≥1.5 standard deviation (SD) lower than the average. Suckling Hu lambs with birth weights near the mean litter birth weight (within 0.5 SD) were deemed to have normal birth weights (CON) (Zhang et al., 2019a). On d 7 after birth, 12 and 36 newborn Hu lambs weighing 4.25 ± 0.14 kg (CON) and 3.01 ± 0.12 kg (IUGR), respectively, were selected from a cohort of 432 twin lambs at the

Jiangyan Experimental Station (Taizhou, Jiangsu, China). Lambs were weaned and separated from their dams at 7 d old and were assigned, based on their initial body weight (BW), to 1 of 4 treatment groups with 12 animals in each group in triplicates with 4 lambs per replicate (2 males and 2 females). The treatment groups were as follows: CON (4.25 ± 0.14 kg), IUGR (3.01 ± 0.12 kg), IUGR + 1% Arg (2.99 ± 0.13 kg), or IUGR + 0.1% NCG (3.03 ± 0.11 kg) (Zhang et al., 2020). Three replications were in each treatment, and 4 lambs were in each replication. The trial lasted for 21 d (until d 28 after birth) during which period lambs in each replicate pen were housed in a 4 m × 1 m indoor pen with free access to clean water and fed on iso-caloric and iso-nitrogenous basal diets of milk replacer (Table 1) to meet nutrient requirements of suckling lambs (NRC, 2007). Nitrogen was equilibrated using L-alanine (Ajinomoto Co., Ltd., Beijing, China). NCG with 97% purity was provided by Sigma−Aldrich Corporation (Louis, Missouri, US) whereas Arg was provided by Ajinomoto Co., Ltd., (Beijing, China). The dose of NCG (0.1%) and Arg (1%) was determined based on previous trials on lambs (Zhang et al., 2018d, 2019b), piglets (Yang et al., 2013) and rats (Cao et al., 2016). The milk replacer feeding rate was adjusted every 10 d at a rate of 2% of the live body weight of each lamb. Before feeding, milk replacer was dissolved in hot water to make a 40 °C solution (containing 16.67% DM), and it was given to every lamb 3 times a day (at 07:00, 13:00 and 19:00). Experienced farm personnel were responsible for feeding the lambs with milk replacer, thus avoiding confounding factors such as daily handling and management. The individual daily intake of milk replacer was calculated as the difference between the offered and refused amounts. The average dry matter intake (ADMI) of milk replacer was also computed daily via multiplying the average daily milk intake by its DM level (%).

2.2. Hepatic sample collection

On d 28 of age (final day of the experiment) and before sacrifice, lambs were anesthetized by intravenous injection of sodium

Table 1
Ingredients and nutrient composition of milk replacer (%, dry-matter basis)[1].

Item	Content
Ingredients	
Whey protein concentrate, 34% CP	30.00
Milk fat powder, 11% CP	35.00
Whole milk powder	20.00
α-Casein	5.00
Glucose	6.00
Pre-mixed mixture[2]	4.00
Nutrient content (analyzed)[3]	
GE, MJ/kg	19.36
CP, %	23.77
EE, %	15.65
Ash, %	7.64
Lysine, %	1.53
Methionine, %	0.41
Threonine, %	0.87
L-Arginine, %	0.63
Ca, %	0.99
TP, %	0.73

GE = gross energy; CP = crude protein; EE = ether extract; Ca = calcium; TP = total phosphorus.

[1] This table is cited from Zhang et al. (2020).

[2] Main contents of the pre-mixed mixture (per kilogram of the pre-mixed mixture): Cu (as $CuSO_4 \cdot 5H_2O$) 600 mg; Mn ($MnSO_4 \cdot H_2O$) 315 mg; Fe ($FeSO_4 \cdot 7H_2O$) 8,400 mg; Zn ($ZnSO_4 \cdot 7H_2O$) 12,500 mg; Se (as Na_2SeO_3) 17 mg; vitamin A 55,000 IU; vitamin E 400 IU; vitamin D 5,500 IU; vitamin K 12.5 mg; biotin 2 mg; folacin 7.5 mg; choline 15 mg; riboflavin 100 mg; vitamin B_6 175 mg; thiamin 317.5 mg; and vitamin B_{12} 500 mg.

[3] Nutrient levels are all measured values.

pentobarbital (15 mg/kg). Then, hepatic tissue was extracted immediately and weighed, frozen promptly in liquid nitrogen, and preserved at −80 °C until further analysis.

2.3. Determination of hepatic adenosine diphosphate (ADP), ATP and adenosine monophosphate (AMP) levels

The hepatic ADP, ATP and AMP levels were determined by high-performance liquid chromatography (HPLC) following the method of Wang et al. (2016). The levels of the total adenine nucleotide (TAN) and adenylate energy charges (AEC) were calculated using the following equations (Zhang et al., 2019b):

$$TAN = ATP + ADP + AMP;$$

$$AEC = (ATP + 0.5\ ADP)/(ATP + ADP + AMP).$$

2.4. Determination of hepatic tricarboxylic acid (TCA) cycle key enzyme activities

The activities of hepatic TCA key enzymes, including isocitrate dehydrogenase, citrate synthase and alpha-oxoglutarate dehydrogenase complex, were determined in hepatic tissue using the commercial ovine enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Yuan ye Biotechnology Co., Shanghai, China) following the manufacturer's instruction. In brief, the standard solution (50 μL) or the supernatant of the liver tissue extract after dilution was added into each well in the plate. Subsequently, horseradish peroxidase (HRP)-conjugated reagent (100 μL) was added and the plates were incubated at 37 °C for 60 min. Thereafter, the plates were rinsed 5 times with a washing solution, followed by the addition of chromogen solutions A (50 μL) and B (50 μL) and then the plates were mixed and incubated again at 37 °C for 15 min. Finally, a stopping solution (50 μL) was added into the plates and the absorbance was read by the ELISA reader at 450 nm (Model 550; Bio-Rad, Hercules, CA, USA) (Zhang et al., 2019, b; Pi et al., 2014).

2.5. Determination of hepatic mitochondrial complex's activities

Mitochondria were isolated from the fresh liver using a tissue mitochondria isolation kit (No. C3606, Beyotime, Nantong, China) following the manufacturer's instruction. Activities of mitochondrial complexes, including nicotinamide-adenine dinucleotide (NADH) ubiquinone reductase (complex I), succinate ubiquinone reductase (complex II), ubiquinol cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV), were detected following the descriptions of Medja et al. (2009) and Hargreaves et al. (2018). All results were normalized to total protein concentration in each sample for inter-sample comparison (Zhang et al., 2018e).

2.6. Assessment of nicotinamide adenine dinucleotide (NAD) and NADH contents in the hepatic homogenate

Hepatic tissue was homogenized using a lysis buffer, followed by 15 min of centrifugation at 15,000 × g and 4 °C. Then, the supernatant was isolated for measuring the levels of NADH and NAD using a NAD/NADH Quantification Kit (No. MAK037, Sigma, MO, USA) (Zhang et al., 2018a).

2.7. Determination of the mitochondrial DNA (mtDNA) copy number

Total DNA in hepatic tissues was separated by a QIAamp DNA Mini Kit (No.51304, QIAGEN, Hilden, Germany), and the mtDNA copy number was quantified with qRT-PCR using the genomic DNA as the loading control (Zhang et al., 2016b). The mitochondrion-coding cytochrome b (cyt b, NCBI: KY662385.1) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH, XM_012166462.1) genes were used to quantify mtDNA and nuclear DNA, separately. The primer sequences are displayed in Table 2. The PCR reaction was prepared with a final volume of 20 μL and performed with a 3-step PCR amplification program. At the end of amplification, the melting curve was analyzed, and the relative mtDNA copy number was measured based on the mtDNA-to-nuclear DNA ratio.

2.8. Real-time PCR for measuring the mRNA expression

Total RNA in hepatic tissues was separated, quantified, and reversely transcribed to the complementary DNA (cDNA) following a previous report (Pi et al., 2014). The expression of the target gene was measured relative to the GAPDH level by the $2^{-\Delta\Delta CT}$ approach (Livak and Schmittgen, 2001). The relative mRNA level of each target gene was standardized to that in the CON group. Table 2 displays the sequences and the GenBank accession numbers of primer sets used to amplify target genes.

2.9. Western blotting

Hepatic protein expression was measured as previously reported (Kang et al., 2015). In brief, an equivalent amount of protein was isolated from the hepatic supernatant solutions by using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was then transferred to the blotting membrane to carry out immunoblotting (Wang et al., 2015). The primary antibodies used in this study included total AMPKα (tAMPKα), rabbit

Table 2
Primer sequences used in the real-time PCR.

Gene	Sequence (5′–3′)	GenBank accession number
AMPKα1	F : GACTGCTACTCCACAGAGATCG	NM006251
	R : TCAGCATCTGAATCACTCCTTT	
AMPKα2	F : TGCGGAGGGCCATTCA	NM023991
	R : TGAGACAGAGGACGACATGCT	
SIRT1	F : CGAGAGGCGAGGAGGAGAAG	XM_015104377.1
	R : ATCGTTCGAGGATCTGTGCC	
SIRT3	F: GCCCAATGCCACTCACTACT	XM_004019744.3
	R: GGGATGCCAGATGCTCTCTC	
PGC1α	F : CACCCACAACTCCTCCTCAT	AY321517.1
	R : CCTTCCTTTCCTCGTGTC	
NRF1	F : CGGAAGAGACAGCAAACACG	AY368269.1
	R : GGTTGGGTTTGGAGGGAGAG	
NRF2	F: CGAGCCGGTGTGAGTAGA	AY369137.1
	R: TTCCGTGGCCCAGTGTAAAG	
ESRRA	F: TGCAGATCACCAAGCGAGAG	XM_015103167.1
	R: GTCTCACCTGTCTTCCGAGG	
TFAM	F: CGCTCCCCCTTTAGTTTTGC	XM_015104510.1
	R: CTGCCAGTCTGCCCTGTAAG	
mtD-loop	F: ACTCGGAGACCCAGACAACT	KY662385.1
	R: TGTAGGGGTGTTCAACTGGC	
GAPDH	F : GTCAAGGCAGAGAACGGGAA	XM_012166462.1
	R : GGTTCACGCCCATCACAAAC	

AMPK = AMP-activated protein kinase; SIRT = silent information regulator; PGC1α = peroxisome proliferator-activated receptor γ coactivator-1α; NRF = nuclear respiratory factor; ESRRA = estrogen-related receptor alpha; TFAM = transcription factor A, mitochondrial; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

phosphorylated AMPKα (pAMPKα or Thr172), SIRT1, PGC1α, NRF1, mouse anti-β-actin (Sigma−Aldrich, Inc.) and TFAM (Cell Signaling Technology Inc., Danvers, MA, USA). All were diluted at a rate of 1:1,000. The secondary antibodies adopted in this study included goat anti-mouse IgG HRP and goat anti-rabbit IgG HRP. Both were diluted at a rate of 1:1,500 (Antgene Biotech). The pAMPKα, tAMPKα, SIRT1, PGC1α, TFAM and NRF1 protein bands were developed by an enhanced chemiluminescence western blotting kit (Amersham), visualized using the Gene Genome Bioimaging System and analyzed through the GeneTools software (Syngene). The phosphorylated AMPKα was standardized according to its expression level, which was the total protein expression of AMPKα.

2.10. Statistical analysis

Results are expressed as means with pooled SEM. The fixed effect of sex was included in the original statistical model but was not significant ($P > 0.05$); thus, it was eliminated from the final model, in which treatment alone was the fixed effect. Data analysis was performed by one-way analysis of variance (ANOVA) using SPSS 10.0 (SPSS, Chicago, IL, USA). Tukey's post hoc test was applied for multiple comparisons among treatment groups. The significance level was set to $P < 0.05$. The data are presented as means and SEM.

3. Results

3.1. Hepatic ADP, ATP and AMP contents

The IUGR lambs experienced a decline in ATP, TAN, ADP, AMP and AEC levels with a rise in the AMP-to-ATP ratio in the liver ($P < 0.05$) compared to the CON lambs (Table 3). Compared to IUGR lambs, lambs that received NCG or Arg supplementation showed an increase ($P < 0.05$) in the contents of ADP, ATP, AMP and TAN with a decrease ($P < 0.05$) of the AMP-to-ATP ratio in the liver. Compared to CON lambs, the contents of ADP, ATP, AMP and TAN were lower ($P < 0.05$) and the AMP-to-ATP ratio was greater ($P < 0.05$) in the liver in lambs that received NCG or Arg supplementation. The hepatic AEC level was similar ($P > 0.05$) between IUGR lambs and those that received NCG or Arg. There were no differences ($P > 0.05$) in the ATP, TAN, ADP, AMP, AEC levels, and AMP-to-ATP ratio in the liver of lambs between NCG and Arg supplementation.

3.2. Activities of key enzymes of hepatic TCA cycle

The IUGR lambs showed a decline ($P < 0.05$) in the hepatic alpha-oxoglutarate dehydrogenase complex, isocitrate dehydrogenase and citrate synthase activities compared with those in the

CON lambs (Table 4). Dietary NCG or Arg supplementation increased ($P < 0.05$) the hepatic alpha-oxoglutarate dehydrogenase complex, isocitrate dehydrogenase, and citrate synthase activities compared to IUGR lambs. Compared to CON lambs, the activities of hepatic alpha-oxoglutarate dehydrogenase complex, isocitrate dehydrogenase, and citrate synthase were lower ($P < 0.05$) in the liver in lambs that received NCG or Arg supplementation. There were no differences ($P > 0.05$) in the hepatic alpha-oxoglutarate dehydrogenase complex, isocitrate dehydrogenase and citrate synthase activities in lambs between NCG and Arg supplementation.

3.3. Hepatic mitochondrial complex's activities

The activities of hepatic mitochondrial complexes I/III/IV were lower ($P < 0.05$) in IUGR lambs than those in the CON group (Table 5). The NCG- or Arg-supplemented lambs had greater and lower ($P < 0.05$) activities of hepatic mitochondrial complexes I/IV compared with those of the IUGR and CON lambs, respectively. Comparing NCG or Arg supplementation with the CON lambs, no difference ($P > 0.05$) was found in the activity of hepatic mitochondrial complex III. Hepatic mitochondrial complex II activity was similar ($P > 0.05$) among treatment groups. No differences ($P > 0.05$) were found in the activities of hepatic mitochondrial complexes I/III/IV in lambs between NCG and Arg supplementation.

3.4. Hepatic NADH and NAD contents

The NAD level and NAD-to-NADH ratio were increased ($P < 0.05$) in the liver of IUGR lambs compared to those of the CON lambs (Table 6). The NAD level and NAD-to-NADH ratio were reduced ($P < 0.05$) in the liver of IUGR lambs treated with NCG or Arg compared to those in IUGR lambs. Compared to CON lambs, the hepatic NAD level and NAD-to-NADH ratio were greater ($P < 0.05$) in lambs that received NCG or Arg supplementation. The hepatic NADH level was similar ($P > 0.05$) among treatment groups. No differences ($P > 0.05$) were found in the NAD level and NAD-to-NADH ratio in the liver of lambs between NCG and Arg supplementation.

3.5. Gene expression

The relative mtDNA copy number and the mRNA expression of AMPKα1, AMPKα2, SIRT1, SIRT3, PGC1α, NRF1 and TFAM were higher ($P < 0.05$) in the liver of IUGR lambs compared to those of the CON ones (Table 7). The relative mtDNA copy number and the mRNA expression of AMPKα2, SIRT1, SIRT3, PGC1α, and NRF1 were lower and greater ($P < 0.05$) in the liver of IUGR lambs treated with NCG

Table 3
Effects of ʟ-arginine or N-carbamylglutamate (NCG) supplementation on adenylate purines in the liver of intrauterine growth retarded suckling lambs[1].

Item	Groups[2]				SEM	P-value
	CON	IUGR	IUGR+1%Arg	IUGR+0.1%NCG		
ATP, μg/g wet wt	108[a]	61.4[c]	84.1[b]	82.0[b]	5.95	0.007
ADP, μg/g wet wt	139	71.1	99.3[b]	103[b]	9.11	0.010
AMP, μg/g wet wt	183[a]	136[c]	151[b]	159[b]	11.24	0.013
AMP-to-ATP ratio	1.69[c]	2.24[a]	1.83[b]	1.92[b]	0.171	0.005
TAN[3], μg/g wet wt	431[a]	269[c]	335[b]	344[b]	14.32	0.009
AEC[4]	0.43[a]	0.35[b]	0.40[ab]	0.39[ab]	0.012	0.021

ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; TAN = total adenine nucleotide; AEC = adenylate energy charge.
[a,b,c] Mean values within a row with different superscript letters were significantly different ($P < 0.05$).
[1] Mean values with their SEM, $n = 12$ in each group.
[2] CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.
[3] TAN = ATP + ADP + AMP.
[4] AEC = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Table 4
Effects of ʟ-arginine or N-carbamylglutamate (NCG) supplementation on TCA cycle key enzyme activities in the liver of intrauterine growth retarded suckling lambs[1].

Item	Groups[2]				SEM	P-value
	CON	IUGR	IUGR+1%Arg	IUGR+0.1%NCG		
Citrate synthase, mIU/g protein	295[a]	209[c]	253[b]	251[b]	18.25	0.008
Isocitrate dehydrogenase, mIU/g protein	49.6[a]	30.2[c]	38.5[b]	38.1[b]	2.79	0.009
Alpha-oxoglutarate dehydrogenase complex, μg/g protein	384[a]	266[c]	312[b]	320[b]	21.96	0.011

[a,b,c] Mean values within a row with different superscript letters were significantly different ($P < 0.05$).
[1] Mean values with their SEM, $n = 12$ in each group.
[2] CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.

Table 5
Effect of ʟ-arginine or N-carbamylglutamate (NCG) supplementation on mitochondrial electron transport chain enzymes (I, II, III and IV) in the liver of intrauterine growth retarded suckling lambs (nmol/mg protein per min)[1].

Item	Groups[2]				SEM	P-value
	CON	IUGR	IUGR+1%Arg	IUGR+0.1%NCG		
Complex I	316[a]	240[c]	274[b]	278[b]	13.89	0.009
Complex II	342	337	340	342	9.75	0.201
Complex III	603[a]	518[b]	594[a]	602[a]	11.25	0.009
Complex IV	309[a]	220[c]	258[b]	260[b]	9.38	0.006

[a,b,c] Mean values within a row with different superscript letters were significantly different ($P < 0.05$).
[1] Mean values with their SEM, $n = 12$ in each group.
[2] CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.

or Arg in comparison to those of the IUGR and CON lambs, respectively. Comparing NCG or Arg supplementation with the CON lambs, no differences ($P > 0.05$) were found in the hepatic mRNA expression of *AMPKα1* and *TFAM*. There were no differences ($P > 0.05$) in the relative mtDNA copy number and mRNA expression of the above-mentioned genes in the liver of lambs between NCG and Arg supplementation.

3.6. Protein expression

The pAMPKα-to-tAMPKα ratio and the protein expression of PGC1α, SIRT1, NRF1 and TFAM were up-regulated in IUGR lambs compared to those of the CON ones ($P < 0.05$) (Fig. 1A and Fig. 2). Compared to IUGR and CON lambs, NCG- or Arg-supplemented lambs had lower and greater ($P < 0.05$) pAMPKα-to-tAMPKα ratio and protein expression of SIRT1, NRF1 and TFAM in the liver, respectively. Comparing NCG or Arg supplementation with the CON lambs, no difference ($P > 0.05$) was found in the PGC1α protein expression. There were no differences ($P > 0.05$) in hepatic PGC1α,

SIRT1, NRF1, TFAM, and tAMPKα protein levels between Arg and NCG treatments (Fig. 1B).

4. Discussion

The liver exerts a vital role in the regulation of whole-body energy metabolism. ATP represents a major energy source in most cell functions, and the level of AEC represents the high-energy phosphate content in the cells (Hardie and Hawley, 2001; Kang et al., 2015). The AMP-to-ATP ratio can be used as a biomarker for intracellular energy status (Hardie and Hawley, 2001). Relative to the individual nucleotide scale, energy charge within the adenyl pool may be potentially used as a favorable approach for measuring the energy status in tissues (Hou et al., 2011). In this study, dietary supplementation of NCG or Arg increased the hepatic ATP and AEC levels but decreased the AMP-to-ATP ratio, which indicated that NCG or Arg enhanced the ATP production to increase the IUGR-mediated energy supply. Our results are consistent with those of Zhang et al. (2019b) who reported that the ATP content in the intestine of IUGR suckling lambs was increased by dietary supplementation of NCG or Arg. These results imply that dietary NCG or Arg administration could mitigate IUGR-induced hepatic mitochondrial dysfunctions through modulating the adenine nucleotide pool (Fig. 3).

The TCA cycle may be used as a critical approach to produce energy in the liver (Kang et al., 2015) via the TCA cycle key enzymes such as isocitrate dehydrogenase, citrate synthase and α-ketoglutarate dehydrogenase complex. The citrate synthase can catalyze the first step in this cycle through the attachment of acetate molecules onto oxaloacetate (Wiegand and Remington, 1986). Isocitrate dehydrogenase is also capable of catalyzing oxidative decarboxylation for isocitrate, which can thereby generate a-ketoglutarate and CO_2 (Corpas et al., 1999). The a-ketoglutarate dehydrogenase complex, the complex constituted by different enzymes, can convert α-ketoglutarate into succinyl-CoA (Corpas et al., 1999). According to our results, dietary supplements of NCG or Arg to IUGR lambs could alleviate the reduced critical enzyme activities during the TCA cycle; this is because

Table 6
Effect of ʟ-arginine or N-carbamylglutamate (NCG) supplementation on NAD and NADH level in the liver of intrauterine growth retarded suckling lambs[1].

Item	Groups[2]				SEM	P-value
	CON	IUGR	IUGR+1%Arg	IUGR+0.1%NCG		
NAD, nmol/mg protein	2.07[c]	5.89[a]	3.86[b]	3.79[b]	0.312	0.006
NADH, nmol/mg protein	1.37	1.41	1.35	1.39	0.061	0.119
NAD-to-NADH ratio	1.51[c]	4.18[a]	2.86[b]	2.74[b]	0.089	0.007

NAD = nicotinamide adenine dinucleotide; NADH = nicotinamide adenine dinucleotide hydride.
[a,b,c] Mean values within a row with different superscript letters were significantly different ($P < 0.05$).
[1] Mean values with their SEM, $n = 12$ in each group.
[2] CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.

Table 7
Effect of ʟ-arginine or *N*-carbamylglutamate (NCG) supplementation on gene expression related to energy metabolism and mitochondrial biogenesis in the liver of intrauterine growth retarded suckling lambs[1].

Item	Groups[2]				SEM	*P*-value
	CON	IUGR	IUGR+1%Arg	IUGR+0.1%NCG		
Relative mtDNA copy number	1.00[c]	2.23[a]	1.52[b]	1.59[b]	0.101	0.006
AMPKα1	1.00[b]	1.89[a]	1.13[b]	1.09[b]	0.082	0.009
AMPKα2	1.00[c]	1.97[a]	1.45[b]	1.51[b]	0.088	0.004
SIRT1	1.00[c]	2.18[a]	1.66[b]	1.64[b]	0.112	0.012
SIRT3	1.00[c]	3.09[a]	2.17[b]	2.11[b]	0.131	0.007
PGC1α	1.00[c]	3.54[a]	2.31[b]	2.29[b]	0.079	0.015
NRF1	1.00[c]	3.04[a]	1.99[b]	2.03[b]	0.062	0.021
NRF2	1.00	0.95	0.99	1.02	0.101	0.209
ESRRA	1.00	1.07	0.96	1.05	0.068	0.117
TFAM	1.00[b]	1.74[a]	1.09[b]	1.11[b]	0.059	0.022

AMPK = AMP-activated protein kinase; *SIRT* = silent information regulator; *PGC1α* = peroxisome proliferator-activated receptor γ coactivator-1α; *NRF* = nuclear respiratory factor; *ESRRA* = estrogen-related receptor alpha; *TFAM* = transcription factor A, mitochondrial.
[a,b,c] Mean values within a row with different superscript letters were significantly different ($P < 0.05$).
[1] Mean values with their SEM, $n = 12$ in each group.
[2] CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.
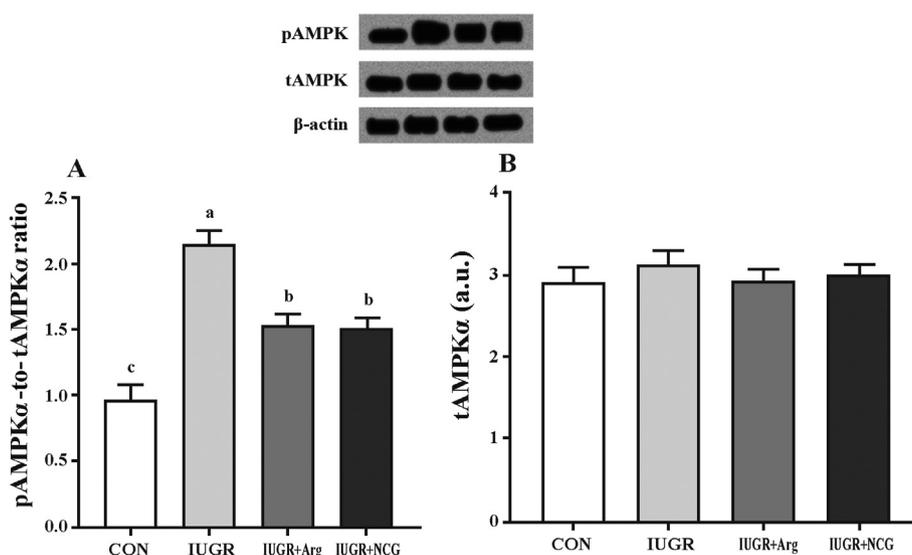
Fig. 1. Effects of ʟ-arginine or N-carbamylglutamate (NCG) supplementation on the phosphorylated AMPKα (pAMPKα)-to-total AMPKα (tAMPKα) ratio (A) and the protein abundance of tAMPKα (B) in the liver of IUGR suckling lambs. The bands shown are the representative western blot images of pAMPKα, tAMPKα and β-actin. Beta-actin was from the same blot as the proteins of interest. Data were analyzed as repeated measures with treatments. Mean values in columns without a common letter differ ($P < 0.05$). Values are means ($n = 12$ per group), with their standard errors represented by vertical bars. a.u. = arbitrary units; AMPK, AMP-activated protein kinase. CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.

NCG or Arg is converted to aspartate, which is further transformed into the TCA cycle intermediates (like oxaloacetate) (Sivakumar et al., 2008). Specifically, the increased oxaloacetate content can enhance citrate synthase activity (Sivakumar et al., 2008). Thus, our results indicate that dietary NCG or Arg supplementation could improve the hepatic energy status by enhancing the activities of the TCA cycle key enzymes. Also, Arg participates in the urea cycle, and the product, fumaric acid, makes a connection between the urea cycle and the TCA cycle to increase oxaloacetate. According to Schanbacher et al. (1981), the serum isocitrate dehydrogenase level was elevated in liver injury in a cattle model. Consistent with the results of liver ATP content of this study, the NCG- or Arg-treated IUGR lambs had higher liver isocitrate dehydrogenase, citrate synthase and α-oxoglutarate dehydrogenase complex activities, which might trigger the TCA cycle to produce more ATP, thus alleviating IUGR-derived metabolic stress.

AMPK has a vital role in the cellular adaptation to nutritional deprivation in vitro, together with physiopathological stresses in vivo (Amaral et al., 2016). Also, AMPK prevents cellular ATP deficiency and mediates cellular responses to metabolic stress, and can be activated at a greater AMP: ATP ratio (Hardie et al., 1998). Accordingly, activated AMPK can prevent excess ATP consumption and elevate ATP generation in response to the limited available energy (Bolster et al., 2002). Among the 7 SIRT subtypes (1 to 7), SIRT1 was the most extensively investigated; it triggers fatty acid oxidation-related gene expression like carnitine palmitoyltransferase 1 (*mCPT-1*) (Lagouge et al., 2006). Also, the transcription coactivator PGC1α can regulate mitochondrial biogenesis and functions (D'errico et al., 2011) and genes of various biological reactions such as adaptive thermogenesis, mitochondrial biogenesis and glucose/fatty acid metabolism (Liang and Ward, 2006). Additionally, AMPK can also boost SIRT1 activity to meet energy
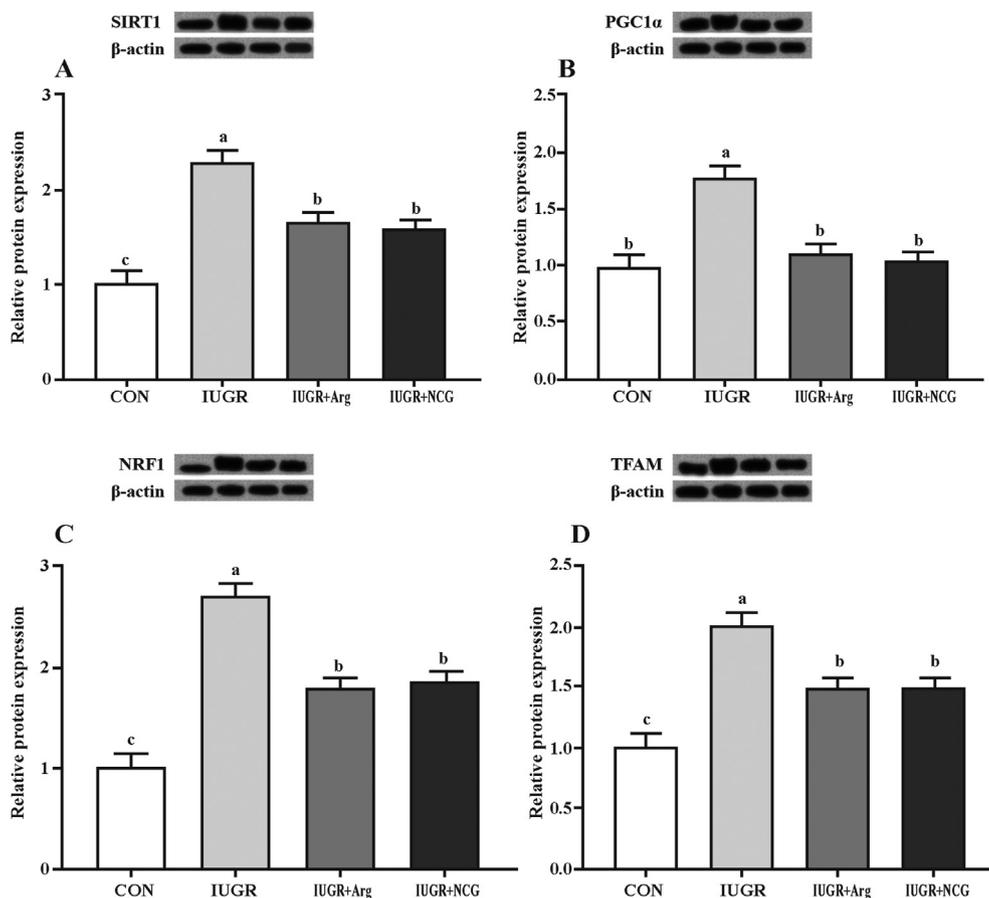
Fig. 2. Effects of ʟ-arginine or N-carbamylglutamate on the protein abundances of silent information regulator 1 (SIRT1) (A), PPARγ coactivator-1α (PGC1α) (B), nuclear respiratory factor 1 (NRF1) (C), and transcription factor A (TFAM) (D) in the liver of IUGR suckling lambs. The bands shown are the representative Western blot images of sirtuin 1 (SIRT1), PGC1α, NRF1, TFAM and β-actin. Beta-actin was from the same blot as the proteins of interest. Data were analyzed as repeated measures with treatments. Mean values in columns without a common letter differ ($P < 0.05$). Values are means ($n = 12$ per group), with their standard errors represented by vertical bars. CON: the normal birth weight group given a control diet; IUGR: intrauterine growth retardation group given a control diet; IUGR+1% Arg: intrauterine growth retardation group given a 1% ʟ-arginine-supplemented diet; IUGR+0.1%NCG: intrauterine growth retardation group given a 0.1% N-carbamylglutamate-supplemented diet.
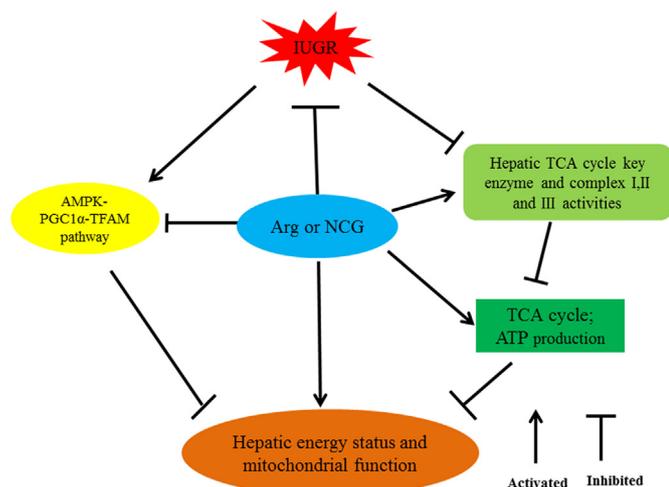
Fig. 3. Possible mechanism of Arg or NCG on the regulation of the hepatic energy status and mitochondrial function in IUGR suckling Hu lambs. Arg = ʟ-arginine; NCG = N-carbamylglutamate; IUGR = intrauterine growth retardation; TCA = tricarboxylic acid cycle; ATP = adenosine triphosphate; AMPK = adenosine monophosphate-activated protein kinase; PGC1α = peroxisome proliferator-activated receptor γ coactivator-1α; TFAM = transcription factor A, mitochondrial.

demand and simultaneously regulate the deacetylation and activation of PGC1α; an essential cell response to the increased mitochondrial metabolism (Fernandez-Marcos and Auwerx, 2011). In the current study, IUGR lambs were found to have elevated expressions of AMPKα2, AMPKα1, PGC1α, and SIRT1 at both mRNA and protein levels with an elevated level of AMPKα phosphorylation. In contrast, dietary NCG or Arg supplementation to our IUGR lambs downregulated the AMPKα2, AMPKα1, PGC1α, and SIRT1 expression in the liver at mRNA and protein levels and reduced the phosphorylation level of AMPKα. Thus, dietary NCG or Arg supplementation inhibited the liver AMPK signal transduction pathway in response to IUGR, which conformed to the reduced AMP-to-ATP ratio and the increased liver ATP level. Possibly, the reduced liver AMP-to-ATP ratio together with the increased liver ATP level might inhibit the AMPK-SIRT1-PGC1α signal transduction pathway in IUGR lambs treated with NCG or Arg.

Mitochondria play a vital role in regulating oxidative stress, and any alteration in them is reported to contribute to cellular adaptation to IUGR in terms of metabolism (Zhang et al., 2017). Activating the AMPK can switch on the ATP-generating catabolic pathway through boosting the mitochondria level (Chen et al., 2013). In this study, IUGR decreased the hepatic mitochondrial complexes I, III and IV activities whereas it up-regulated the PGC1α expression, the downstream AMPK and NRF1 and TFAM expression (the targets of PGC1α). Also, IUGR promoted efficient electron

transport in mitochondria based on the fact that low-potential mitochondria maintained low levels of ATP production, oxygen consumption and ROS production (Bisht and Dada, 2017). In this study, IUGR lambs treated with dietary NCG or Arg supplements efficiently restored the hepatic mitochondrial complex activities, the expression of mitochondrial regulators (*PGC1α, NRF1* and *TFAM*) and the mtDNA copy number; dietary Arg or NCG supplementation restored the *PGC1α* expression, a key factor that coactivated NRF1. The NRF1 can modulate TFAM, while the latter can, in turn, control the transcription and duplication of mitochondrial DNA (Hock and Kralli, 2009). These findings suggested that dietary Arg or NCG supplementation counteracted the increase in NRF1 and TFAM expression levels caused by IUGR.

Sirtuins represent the NAD-dependent protein deacetylases family. SIRT1 is the first gene identified of this family and plays a leading role in energy homeostasis as well as being able to activate the downstream AMPK through modulating the NAD content, the SIRT1 substrate (Canto et al., 2009). According to our results, the mRNA and protein abundance of SIRT1 was up-regulated, but the complex I activity was decreased, and the NAD-to-NADH ratio was elevated in IUGR lambs. After dietary Arg or NCG supplementation, the mRNA and protein abundance of SIRT1 and NAD-to-NADH ratio were down-regulated. Additionally, SIRT1 has the functions of binding, deacetylating, and activating PGC1α, which can thereby promote mitochondrial biogenesis (Rodgers et al., 2005). On the other hand, SIRT3, another SIRT family member, was up-regulated responding to IUGR, and SIRT3 knockdown reduced the AMPK phosphorylation and PGC1α expression, suggesting that SIRT3 has a vital role in regulating energy metabolism (Palacios et al., 2009). These findings further demonstrated that IUGR efficiently promoted mitochondrial biogenesis, thus adapting to metabolic stresses.

5. Conclusion

Dietary supplementation of NCG or Arg improved hepatic energy status and mitochondrial function and inhibited the AMPK-PGC1α-TFAM pathway in IUGR suckling Hu lambs. The above findings can potentially recommend NCG or Ag as a dietary treatment for IUGR-induced hepatic mitochondrial dysfunctions. Importantly, our findings can offer valuable clues to enhance our understanding of the elevated hepatic ATP production and mitochondrial biogenesis in Arg- or NCG-treated IUGR lambs, and further contribute to developing a novel nutritional strategy to mitigate the postnatal energy deficiency in IUGR infants.

Author contributions

Hao Zhang and **Hongrong Wang** designed the research; **Hao Zhang**, **Xiaoyun Liu** and **Shengnan Ren** conducted the research; **Mengzhi Wang** and **Hao Zhang** analyzed the data; **Hao Zhang** and **Mabrouk Elsabagh** wrote the paper; **Hao Zhang** and **Hongrong Wang** had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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